TPLE 1452

Molecular Interactions in β-Lactoglobulin. IV. The Dissociation of β-Lactoglobulin below *p*H 3.5[2]

Ultracentrifugal and light scattering studies have been carried out on β-lactoglobulin between *p*H 1.6 and 5.0. Below *p*H 3.5, β-lactoglobulin undergoes a reversible dissociation due to non-specific electrostatic repulsion between sub-units of 18,000 molecular weight. These sub-units are nearly identical and correspond to the two polypeptide chains of this protein.

Introduction

The generally accepted value of the molecular weight of β-lactoglobulin is 35,000–36,000. Measurements reported in the literature by the techniques of osmotic pressure,[4] X-ray diffraction,[5] sedimentation–diffusion[6] and light scattering[7] agree on a value close to 35,500. In the course of a thorough study of the association properties of this protein as a function of *p*H, it has been found that the state of aggregation of its dispersed units is strongly dependent on *p*H. In the previous two papers[8,9] it has been shown that in the *p*H region (5.2–3.7) immediately below its isoelectric point (5.2–5.3), part of β-lactoglobulin undergoes a reversible tetramerization at temperatures close to zero. If the *p*H is decreased still further to below 3.5, this protein has been found to undergo another reaction,[10,11] in this case a dissociation into smaller molecular sub-units. It is the purpose of this paper to present the detailed light scattering and ultracentrifugal studies of the nature and stoichiometry of this dissociation.

Experimental

Materials.—The protein used in this study was a β-lactoglobulin prepared from pooled milk and referred to as "Prep II" in the previous papers.[8,9,12]

Methods.—Most ultracentrifuge experiments were carried out on a Spinco Model E[13] analytical ultracentrifuge at 59,780 r.p.m., equipped with a phase plate and a temperature control unit. Some low concentration runs were carried out at 50,740 r.p.m. in the B rotor with a cell thickness of 30 mm. Sedimentation constants were measured with a microcomparator. Archibald sedimentation runs[14] were carried out according to the method of Brown,[15,16] the data being analyzed from enlarged projected tracings of the patterns. The total protein concentration was obtained from the area under the pattern formed in a synthetic boundary cell.[17,18] In all cases, the proper dilution correction[19] was applied to the patterns, segment by segment.

Light scattering measurements were carried out on the Brice photometer[20] at 436 mμ using 2 mm. slit optics. Solutions were clarified by filtration in an ultrafine sintered glass filter of special design.[21,22] All measurements were performed in 1 cm. square (3 ml.) cells starting with pure solvent and slowly adding increments of concentrated stock solution, according to the Dintzis technique.[23]

Protein solutions were prepared by dissolving three times recrystallized β-lactoglobulin in a 0.1 ionic strength solution of NaCl and adjusting the *p*H to the desired value with 0.1 *M* HCl or NaOH. This solution was then dialyzed against a 0.1 ionic strength NaCl–HCl or NaCl–NaOH solution of identical *p*H. Concentrations were measured by ultraviolet absorption at 278 mμ, using a value of 0.96 l./cm.g. for the absorptivity.[8] Stock solution concentrations were 80–100 g./l. The value of the refractive index increment used was 0.1890.[7] All *p*H's were measured on a Beckman Model G *p*H meter[13] at 25°.

Results

The dependence of the sedimentation constant on *p*H at 25° for solutions of β-lactoglobulin (9–12 g./l.) is shown in Fig. 1. It can be seen that while values of the sedimentation constant are normal[8] (2.9 *S*) in the isoelectric region, at *p*H's near 4 $s_{20,w}$ starts to drop until at *p*H 2.0, it has reached a value of *ca.* 2.3 *S*. Further decrease in *p*H appears to result in no additional change.

The concentration dependences of the sedimentation constants at *p*H's 5.2, 3.5 and 1.6 are shown in Fig. 2. At *p*H 5.2, the dependence is of the usual type, having a slight negative slope. The best straight line through the points follows the equation

$$s_{20,w} = (3.04 - 0.016\,c) \times 10^{-13} \qquad (1)$$

which is essentially that found[24] for this protein

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

(2) This work was presented in part at the 130th National Meeting of the American Chemical Society, Atlantic City, September 1956 and at the 131st Meeting, Miami, April, 1957.

(3) This paper is taken in part from the dissertation to be submitted by Robert Townend to the faculty of Temple University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(4) H. B. Bull and B. T. Currie, THIS JOURNAL, **68**, 742 (1946).

(5) F. R. Senti and R. C. Warner, *ibid.*, **70**, 3318 (1948).

(6) R. Cecil and A. G. Ogston, *Biochem. J.*, **44**, 33 (1949).

(7) M. Halwer, G. C. Nutting and B. A. Brice, THIS JOURNAL, **73**, 2786 (1951).

(8) R. Townend, R. J. Winterbottom and S. N. Timasheff, *ibid.*, **82**, 3161 (1960).

(9) R. Townend and S. N. Timasheff, *ibid.*, **82**, 3168 (1960).

(10) R. Townend and S. N. Timasheff, *ibid.*, **79**, 3613 (1957).

(11) D. G. Rands, Doctoral Dissertation, State University of Iowa, 1957.

(12) S. N. Timasheff and R. Townend, THIS JOURNAL, **82**, 3157 (1960).

(13) Mention of specific firms and products does not imply endorsement by the Department to the possible detriment of others not mentioned.

(14) W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204 (1947).

(15) R. A. Brown, D. Kritchevsky and M. Davies, THIS JOURNAL, **76**, 3342 (1954).

(16) R. A. Brown, M. Davies, M. Englert and H. R. Cox, *ibid.*, **78**, 5077 (1956).

(17) E. G. Pickels, W. F. Harrington and H. K. Schachman, *Proc. Natl. Acad. Sci., U. S.*, **38**, 943 (1952).

(18) S. M. Kleiner and G. Kegeles, *J. Phys. Chem.*, **59**, 952 (1955).

(19) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford, 1940.

(20) B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950).

(21) F. F. Nord, M. Bier and S. N. Timasheff, THIS JOURNAL, **73**, 289 (1951).

(22) M. Bier, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Academic Press, Inc., New York, N. Y., 1957, p. 165.

(23) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, THIS JOURNAL, **79**, 782 (1957).

(24) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

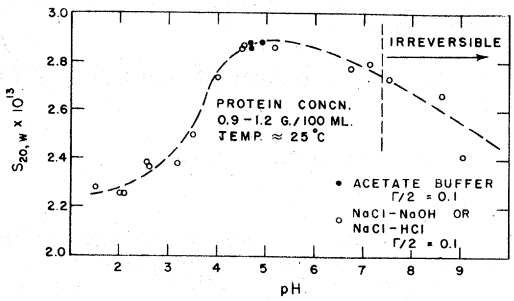

Fig. 1.—pH dependence of $s_{20,w}$ of β-lactoglobulin: 9–12 g./l. protein; 25°; O, NaCl–NaOH or NaCl–HCl, $\Gamma/2$ = 0.1; ●, acetate buffer, $\Gamma/2$ = 0.1.

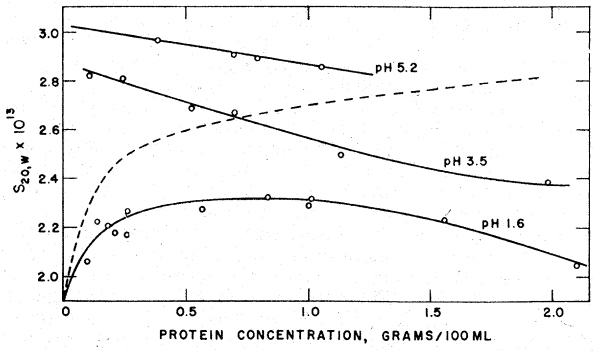

Fig. 2.—Concentration dependence of $s_{20,w}$ of β-lactoglobulin at various pH's. Dashed line, calculated according to Gilbert theory for pH 1.6 from light scattering data.

under conditions where neither aggregation nor dissociation occurs. At pH 3.5 the negative slope is steeper and seems to extrapolate to around 2.9 S. At pH 1.6 the situation is much more complicated. As the concentration decreases from 20 g./l., $s_{20,w}$ first increases, passes through a maximum at 5–10 g./l. and then decreases again, tending toward a value of near 1.9 S at zero concentration.

A pH dependence of the sedimentation constant such as shown in Fig. 1 can be due to one of two causes or a combination of the two. First, this could be due to a change in the shape of the protein molecule resulting in an increase in the frictional coefficient, as has been found with serum albumin,[25–28] which is known to "expand" at low pH's.[29,30] The other possibility is that the molecule dissociates into smaller kinetic units at lower pH, which would also lead to a decrease in sedimentation constant in accordance with the Svedberg equation[19]

$$s = \frac{M(1 - \bar{V}\rho)}{f} \qquad (2)$$

where M is the molecular weight of the protein, $\bar{V}$ is its partial specific volume, ρ is the solvent density and f is the frictional coefficient. Either an increase in f or a decrease in M would produce

(25) P. Bro, S. J. Singer and J. M. Sturtevant, THIS JOURNAL, **77**, 4924 (1955).

(26) M. J. Kronman, M. D. Stern and S. N. Timasheff, *J. Phys. Chem.*, **60**, 829 (1956).

(27) S. N. Timasheff and R. J. Gibbs, *Arch. Biochem. Biophys.*, **70**, 547 (1957).

(28) M. J. Kronman and J. F. Foster, *ibid.*, **72**, 205 (1957).

(29) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(30) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

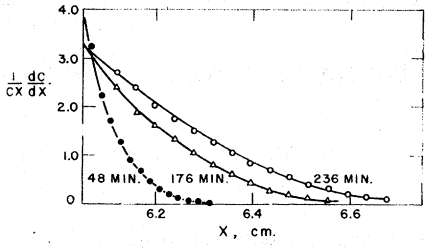

Fig. 3.—Archibald sedimentation data of β-lactoglobulin at pH 2.0, 25°.

the effect observed in Fig. 1. A similar effect would be also observed with any increase in $\bar{V}$, which could also reflect a molecular expansion of a type in which solvent is excluded from the expanded structure. It would be expected that in the case of a molecular expansion the sedimentation constant would extrapolate to a low value but that its concentration dependence would be of the normal type, *i.e.*, decreasing monotonically with increasing concentration. It might be possible, however, to obtain a dependence such as found at pH 1.6 in the case of a flexible polyelectrolyte, where chain flexibility and degree of coiling can be highly concentration dependent as a result of a high charge density on the molecule. At pH 1.6, a β-lactoglobulin molecule 35,500 in molecular weight has a net average charge of +39.[31,32]

Expansion of a protein molecule results in an increase in its intrinsic viscosity.[29,30] β-Lactoglobulin was found in the present study to have identical intrinsic viscosity values of 0.029 at pH 2.0 and 5.2, while optical rotation measurements gave values of α_D of −28.0 and −28.9 for these two pH's. These are strong indications against any gross shape differences at the two pH's. Likewise, Tanford[33] has found essentially no change in intrinsic viscosity over the entire pH range studied in this paper. In view of the above, it seems quite likely that the curve of Fig. 1 at pH 1.6 indicates a dissociation.

In order to establish the degree of dissociation Archibald sedimentation experiments were carried out at pH's between 1.6 and 2.1. The data were analyzed in the cases where the concentration of residual protein at the upper meniscus had become very low, so that the protein would be essentially dissociated. Extrapolation of the data to the upper meniscus at different times in the course of a run is shown in Fig. 3 for a run at pH 2.0. Since the value of $1/cx\ dc/dx$ (where c is the protein concentration and x is the distance from the center of rotation) extrapolated to the meniscus is proportional to the molecular weight, the plots of Fig. 3 demonstrate that the molecular weight of this protein decreases with an increase in the length of the run and, therefore, with a decrease in concentration. Values of the molecular weights obtained in this manner at various pH's and concentrations[10] tend toward 17,000–18,000 as the concentration approaches zero. This indicates that at low pH's

(31) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(32) Y. Nozaki, L. G. Bunville and C. Tanford, THIS JOURNAL, **82**, in press (1960).

(33) C. Tanford, private communication.

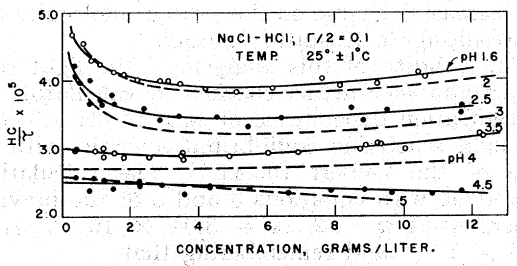

Fig. 4.—Light scattering data of β-lactoglobulin in acid *p*H. 0.1 ionic strength NaCl–HCl, 25°. Dashed lines represent best curves drawn through points at intermediate *p*H's.

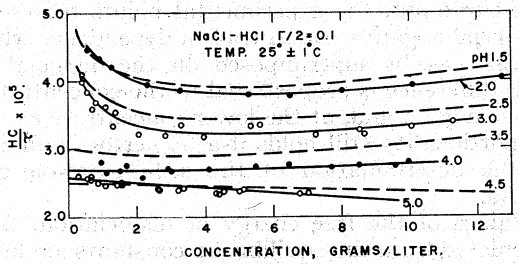

Fig. 5.—Light scattering data of β-lactoglobulin in acid *p*H. 0.1 ionic strength NaCl–HCl, 25°. Dashed lines represent best curves drawn through points at intermediate *p*H's.

β-lactoglobulin undergoes a reversible dissociation into units which are half of the isoelectric kinetic unit.

The fact that in a dissociating system only a single peak is present in the ultracentrifuge is further strong evidence for the presence of a monomer–dimer equilibrium, as has been demonstrated by Gilbert.[34]

Studies on the reversibility of this reaction showed that the dissociation is rapidly and fully reversible. Samples which had been exposed to *p*H 2 could be readily crystallized and their electrophoretic and ultracentrifugal patterns at *p*H 4.6 and 5.2 did not differ from those of the original protein.

In order to characterize the thermodynamics of this dissociation, a light scattering study was carried out between *p*H 1.6 and 5.0, as a function of *p*H, ionic strength and dielectric constant. The results are presented in Figs. 4, 5 and 6. At *p*H's 1.6–3.5 (Figs. 4 and 5) the function $HC_2/\Delta\tau$ first decreases with a decrease in concentration, then, after passing through a minimum at 3–5 g./l., starts to increase. At *p*H 3.5, this effect is very weak, while above *p*H 4.0, it disappears and the data seem to fall on straight lines which extrapolate to molecular weight values in the vicinity of 37,000. At *p*H 4.0 the slope is weakly positive and at *p*H 4.5 and 5.0, weakly negative.

The data obtained at *p*H 2.75 as a function of ionic strength and dielectric constant (Fig. 6) present a similar pattern. The points at the ionic strength of 0.03 pass through a minimum at 4 g./l. but do not deviate strongly from the extrapolated value over the concentration range studied, indicating a very strong dissociation. Data at $\Gamma/2 = 0.1$ behave in a similar way but the points lie in

(34) G. A. Gilbert, *Discussions Faraday Soc.*, No. 2, 68 (1955).

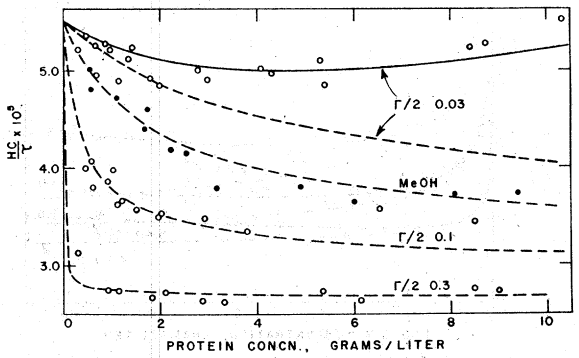

Fig. 6.—Light scattering data of β-lactoglobulin at *p*H 2.7, as a function of ionic strength and dielectric constant. Dashed lines are calculated from equilibrium constants.

general at much lower values of $HC_2/\Delta\tau$. At $\Gamma/2 = 0.3$, the data seem to fall on a horizontal straight line corresponding closely to the isoelectric value of the molecular weight. Data obtained in the presence of 30% methanol (to decrease the dielectric constant of the medium to 69) decrease monotonically with increasing concentration. The low concentration values can be extrapolated to a molecular weight value of 18,000.

Discussion

These data indicate that β-lactoglobulin dissociates at *p*H's below 3.5, the dissociation being enhanced by decrease in *p*H. The light scattering curves seem to tend toward an extrapolated molecular weight of 18,000 suggesting that the dissociation results in half-molecules at low *p*H. The results of the Archibald sedimentation analysis establish this quite conclusively. One should also note that Bull[35] has reported a molecular weight of 17,000 from surface films spread over ammonium sulfate.

Equilibrium constants were calculated from the light scattering data for the reaction $L \rightleftarrows 2L_{1/2}$ using methods described in the previous paper.[8] At any given protein concentration, C_2 (g./l.), in a dimerizing system, the light scattering data are related to the equilibrium dissociation constant K_d, by

$$H\frac{C_2}{\Delta\tau} = \frac{1}{M_{1/2}}[1 + 2B_0C_2] + \frac{1 - \overline{M_w}/M_{1/2}}{\overline{M_w}} \quad (3)$$

$$K_d = \frac{2C_2(2M_{1/2} - \overline{M_w})^2}{M_{1/2}^2\,\overline{M_w} - M_{1/2}^3}$$

where $M_{1/2}$ is the molecular weight of the species with a molecular weight equal to half of the isoelectric value and $\overline{M_w}$ is the weight average molecular weight of the protein system at the given protein concentration.

Using equation 3, equilibrium constants were calculated from the light scattering data below a concentration of 2 g./l. At such a concentration, the product $2B_0C_2$ should be very small and in most cases can be set equal to zero without any significant error. Using the values of the equilibrium constants obtained below 2 g./l., weight-average molecular weights were calculated for various protein concentrations over the entire range covered

(35) H. B. Bull, This Journal, 68, 745 (1946).

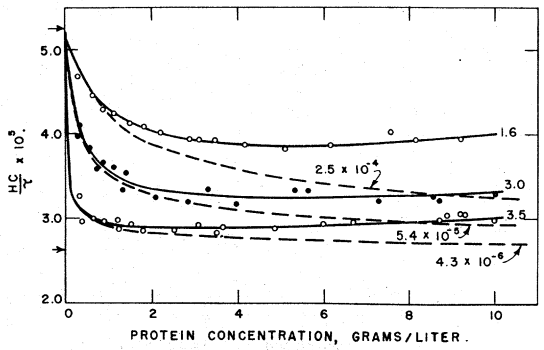

Fig. 7.—Analysis of light scattering data of β-lactoglobulin in acid *p*H. Dashed lines are calculated from equilibrium constants. Solid lines are sum of dashed lines with $(2B_0/M_{1/2})C_2$. *p*H's and values of equilibrium constants are indicated on each curve.

in the experiment. The reciprocals of these values are shown by the dashed lines of Fig. 7, for *p*H 1.6, 3.0 and 3.5. The difference between values on the dashed line and the individual experimental points is equal to $2B_0C_2/M_{1/2}$ from which the second virial coefficient $2B_0/M_{1/2}$ can be obtained. Since this is a constant independent of concentration, the criterion chosen for proper analysis of the data was the calculation of a constant value of $2B_0/M_{1/2}$ over the entire concentration range. The solid lines of Fig. 7 were obtained by multiplying the average value of $2B_0/M_{1/2}$ by the protein concentration and adding the product to the dashed lines. Comparison with the experimental points (Fig. 7, open and filled circles) shows that the values of the equilibrium constants cannot be far wrong. Data obtained at *p*H 2.7 as a function of ionic strength and dielectric constant were analyzed in the same manner. The resulting values of $2B_0/M_{1/2}$ at various conditions are listed in Tables I and II.

TABLE I

DISSOCIATION OF β-LACTOGLOBULIN AT LOW *p*H'S, Γ/2 = 0.1

*p*H	ΔF^0	$\Delta(\Delta F^e)$ (kcal./mole)	$-\Delta F^a$	$2B_0/M_{1/2}$ (l./g.)
1.6	4.9 ± 0.1	−14.1	19.0	12.3×10^{-7}
2.0	4.9 ± .1	−13.6	18.5	7.5×10^{-7}
2.5	5.5 ± .2	−10.8	16.3	5.0×10^{-7}
2.7	5.7 ± .1	− 9.6	15.3	4.6×10^{-7}
3.0	5.8 ± .1	− 7.9	13.7	4.1×10^{-7}
3.5	7.3 ± .2	− 4.8	12.1	3.2×10^{-7}

TABLE II

DISSOCIATION OF β-LACTOGLOBULIN AT *p*H 2.7

Γ/2	ΔF^0	$\Delta(\Delta F^e)$ (kcal./mole)	$-\Delta F^a$	$2B_0/M_{1/2}$ (l./g.)
0.03	3.9 ± 0.1	−17.0	20.9	11.6×10^{-7}
.10	5.7 ± .1	− 9.6	15.3	4.6×10^{-7}
.30	8.3 ± .2	− 7.9	16.2	~0.0
.10*	5.0 ± .1	−11.2	16.2	~0.0

* In 30% methanol, D = 69.

The magnitudes of the second virial coefficients at various *p*H's are reasonable and similar to those of other proteins at like conditions of charge density and ionic strength.[11,23,36] The increasingly positive values of the slopes with decreasing *p*H reflect the increasing charge on the protein molecules and the resulting electrostatic repulsion.

The validity of this analysis of the light scattering data was further checked by calculating the concentration dependence of $s_{20,w}$ at *p*H 1.6 from the light scattering equilibrium constant with the help of the Gilbert theory.[34] The calculations were done with equations 3 and 5 of the previous paper,[8] using $n = 2$, $s_A = 3.04 \times 10^{-13}$, $s_M = 1.90 \times 10^{-13}$ and remembering that

$$K_G = K_d M_{1/2}{}^{n-1}/n \qquad (4)$$

The dashed line in Fig. 2 represents the curve calculated for *p*H 1.6. The difference between this curve and the experimental points represents the usual negative concentration dependence which in this case is superimposed on the dissociation. This difference is proportional to the concentration above 5 g./l., and, at the lower concentrations, the proportionality still holds if a 5% error is allowed in the determination of the sedimentation constants.

Values of the free energy of dissociation, ΔF°, calculated from the equilibrium constants are listed in Tables I and II. The increase of ΔF° with increasing *p*H and ionic strength and its decrease with a decrease in dielectric constant point very strongly to the action of electrostatic forces in this dissociation. The identity of ΔF° at *p*H's 1.6 and 2.0 further supports this, since in that *p*H range the charge on the molecule remains essentially unchanged.[32]

The molar electrostatic free energy of a spherical protein molecule with uniform charge distribution is given by the Linderstrøm-Lang equation[37]

$$\Delta F^e = \frac{Ne^2\bar{Z}^2}{2D}\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \qquad (5)$$

where N is Avogadro's number, e is the electronic charge, $\bar{Z}_2$ is the net average charge on the molecule, D is the dielectric constant of the medium, b is the radius of the protein molecule, κ is the Debye–Hückel screening factor and a is the radius of exclusion of small ions. For a dissociation reaction, the change in electrostatic free energy, $\Delta(\Delta F^e)$, is given by the difference between $\Delta F^e{}_d$ of the dissociated species times the number of particles n formed from one aggregate and $\Delta F^e{}_n$ of the aggregated species

$$\Delta(\Delta F^e) = n\Delta F^e{}_d - \Delta F^e{}_n \qquad (6)$$

Calculation of $\Delta(\Delta F^e)$ for the splitting of β-lactoglobulin ($n = 2$) according to equations 5 and 6 has resulted in the values given in column 3 of Tables I and II. The amount of energy necessary to overcome the attractive force holding the two half molecules together is the difference between the free energy of dissociation and the change in the electrostatic free energy during dissociation. Values of the free energy of attraction, ΔF^a, are given in column 4 of Tables I and II. They are on the order of −12 to −19 kcal./mole. The trend with *p*H, if real, might reflect a molecular rearrangement accompanying dissociation. These values are of course highly approximate as a result of the inherent errors in the analysis of the

(36) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(37) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924).

light scattering data and the nature of the calculations of ΔF^e, in which it was assumed that binding of ions other than hydrogen and hydroxyl does not occur. Furthermore, a charge distribution other than the uniform one implicit in eq. 5 could result in serious changes in the values.[38] The magnitude of the free energy of attraction seems reasonable and could be accounted for by the presence of hydrophobic bonds. Preliminary solubility measurements of β-lactoglobulin in dimethylformamide are compatible with the concept that on dissociation of this protein a hydrophobic surface becomes exposed to the surrounding medium.

Comparison of the sedimentation constants of the isoelectric and dissociated species at infinite dilution (3.04 and 1.90 *S*) with their molecular weights (36,000 and 18,000) gives frictional coefficient ratios, f/f_0, of 1.20 and 1.16, respectively. If the hydration of β-lactoglobulin is taken to be 40%,[39,40] the axial ratios, a/b, for prolate ellipsoids of revolution are then 2.0:1 and 1.1:1, respectively. This is in excellent agreement with the X-ray data of Green and Aschaffenburg,[41] who found that both genetic species of β-lactoglobulin[42] are composed of nearly spherical sub-units of 18,000 molecular weight which are associated into a double molecule of axial ratio 2:1. Their suggestion that a deviation from spherical of the subunit (35.8 Å. in diameter) by 2.3 Å. in one direction would make the molecular packing more reasonable yields a monomer (18,000) molecule with an axial ratio of 1.1:1.

From these data it seems reasonable to suggest that the isoelectric molecule of each type of β-lactoglobulin is made up of two nearly identical halves of *ca.* 18,000 molecular weight. Further strongly suggestive evidence of this is Tanford's finding that the two genetic species differ by two carboxyl groups per 35,500 molecular weight (β-A having two more than β-B),[43] while in all other respects the two proteins seem to be identical. Examining further the chemical properties of the 35,500 units, it is found that all ionizable groups are present in even numbers,[32] there are two abnormal carboxyl groups,[44,45] the molecule seems to be made up of two chains with identical end groups (two N-terminal leucine residues,[46] two C-terminal isoleucine residues[47]); there are two available sulfhydryls[48]; the protein also interacts stoichiometrically with two molecules of sodium dodecyl sulfate,[49] two of *n*-octylbenzene-*p*-sulfonate[50] and two ions of cadmium.[41]

The finding of Tanford and co-workers[43] that β-lactoglobulin A has two more carboxyls than β-lactoglobulin B per 36,000 molecular weight suggests that the electrostatic free energy change in the dissociation should be slightly different for the two genetic species. This would result in a small difference between the free energies of dissociation of the two species. In that event the equilibrium constants reported in Tables I and II would be average values. However, these should not differ greatly from the individual constants of the two β-lactoglobulins. Work is presently in progress to ascertain whether any differences can be detected between the participation of the two genetic species[42] in this reaction.

(38) C. Tanford, THIS JOURNAL, **79**, 5340, 5348 (1957).

(39) D. W. Green, A. C. T. North and R. Aschaffenburg, *Biochim. Biophys. Acta.*, **21**, 583 (1956).

(40) T. L. McMeekin and R. C. Warner, THIS JOURNAL, **64**, 2393 (1942).

(41) D. W. Green and R. Aschaffenburg, *J. Molecular Biology*, **1**, 54 (1959).

(42) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955).

(43) C. Tanford and Y. Nozaki, *J. Biol. Chem.*, **234**, 2874 (1959).

(44) C. Tanford, L. G. Bunville and Y. Nozaki, THIS JOURNAL, **81**, 4032 (1959).

(45) H. Susi, T. Zell and S. N. Timasheff, *Arch. Biochem. Biophys.*, **85**, 437 (1959).

(46) H. Fraenkel-Conrat, "Symposium on Structure of Enzymes and Proteins," Gatlinburg, Tenn., 1955, p. 153.

(47) C. I. Niu and H. Fraenkel-Conrat, THIS JOURNAL, **77**, 5882 (1955).

(48) J. Fraenkel-Conrat, B. B. Cook and A. F. Morgan, *Arch. Biochem. Biophys.*, **35**, 157 (1952).

(49) T. L. McMeekin, B. D. Polis, E. S. Della Monica and J. H. Custer, THIS JOURNAL, **71**, 3606 (1949).

(50) R. M. Hill and D. R. Briggs, *ibid.*, **78**, 1590 (1956).